

Isolation and characterization of two cytochrome P450 aromatase forms in killifish (*Fundulus heteroclitus*): Differential expression in fish from polluted and unpolluted environments

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Abstract

Populations of killifish (*Fundulus heteroclitus*) persist in many different highly polluted environment indicative of adaptation or tolerance. In this study, we sought to determine whether long term, multigenerational exposures to environmental contaminants has affected reproductively relevant genes and biological processes. A homology cloning strategy was used to isolate the killifish cytochrome P450 aromatase (P450arom, estrogen synthetase) cDNAs. Consistent with previous fish studies, killifish were found to have two P450arom forms, which segregated into A- and B-gene clades and were differentially expressed in brain ($B \gg A$) and ovary ($A \gg B$). Comparison of killifish from highly polluted (New Bedford Harbor, NBH) and unpolluted (Scorton Creek, SC) environments revealed no site-related differences in P450arom coding sequences or in overall tissue distribution patterns. As measured by real-time quantitative PCR (QPCR) analysis, however, P450aromB (a known marker of estrogen effect) was approximately two-fold higher in the brain of NBH than of SC fish, a difference seen in reproductively active and inactive males and females. Providing further evidence of exposure to estrogen-like pollutants or metabolites in NBH, vitellogenin (vtg) mRNA and protein were elevated in seasonally active and inactive males, and in reproductively inactive females, when compared to SC fish. By contrast, during the period of reproductive activity, NBH females had a lower gonadosomatic index, lower plasma estrogen, a decreased hepatosomatic index, and reduced vtg expression as compared to SC females, indicating that the female hypothalamic-pituitary-gonadal (HPG)-liver axis is impaired in the polluted environment. As measured by a decrease in plasma androgen (but not GSI), the male HPG axis was impaired in reproductively active NBH versus SC fish. In agreement with reports that NBH killifish are resistant to dioxin-like chemicals (DLC) that activate arylhydrocarbon receptor (AhR) signaling, ovarian P450aromA (a marker of dioxin-like effect in zebrafish embryos) did not differ in SC and NBH fish. In conclusion, the killifish population at the NBH Superfund site maintains a level of reproductive competence in the face of evidence of exposure to estrogen-like pollutants and endocrine disruption.

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1. Introduction

By virtue of their ability to interact with nuclear hormone receptors, many different environmental chemicals have the potential to disrupt critical hormone-regulated processes of reproduction and development, even at low concentrations and transient exposures (Phillips and Harrison, 1999). Endocrine disrupting chemicals (EDCs) have been linked to a range of effects on reproductive health in humans (Phillips and Harrison, 1999) and in animals in the natural environment (Crisp et al., 1998; Phillips and Harrison, 1999; McLachlan, 2001), but epidemiological and wildlife studies rarely are able to establish causality. On the other hand, although laboratory studies in many different species provide support for the endocrine disruptor hypothesis, the applicability of these findings to actual problems of reproduction and development in nature is unclear. One complicating factor is that pollutants are most often present as complex mixtures, with additive, synergistic or antagonistic properties. Second, neither correlational nor experimental approaches per se address the ultimate biological consequences of multi-generational exposures for populations of animals living long term in polluted environments, and only rarely have physiological or genetic adaptations that affect reproductive success of these populations been investigated.

The killifish (*Fundulus heteroclitus*) population at the New Bedford Harbor (NBH) Superfund site in Massachusetts provides a model to evaluate mechanisms by which multi-generational exposure to pollutants may affect reproduction. These non-migratory fish are resident in one of the most extensively polychlorinated biphenyl (PCB)-contaminated estuarine sites in the country (Weaver, 1983). The history of pollution in NBH indicates that the killifish population has survived exposure to high levels of PCBs, dioxin-like chemicals (DLC), heavy metals, and other contaminants for >50 year (15–20 generations). Despite high PCB body burdens the population is able to survive, reproduce and develop under conditions that are toxic in experimentally dosed laboratory or reference site fish (Black et al., 1998a,b). NBH killifish, and wild populations at several other highly polluted sites, have been very well studied as regards toxic responses and phenotypic changes mediated by arylhydrocarbon receptor (AhR) signaling pathways (Wirgin and Waldman, 2004). These fish are

reported to display tolerance or resistance to treatment with DLC (Nacci et al., 1999), as measured by reduced early life stage mortality (Nacci et al., 1999) and altered expression and induction of P4501A1 (Nacci et al., 1999; Bello et al., 2001). There is evidence for heritability of some traits (Nacci et al., 1999; Powell et al., 2000).

AhR are expressed in reproductively relevant tissues such as the brain and gonads of mammals (Matikainen et al., 2002) and killifish (Karchner et al., 1999). In addition to potential direct involvement of AhR in controlling processes of reproduction and development (Abbott et al., 1999; Benedict et al., 2000), estrogen receptor (ER)- and AhR-signaling pathways converge at multiple points (Brunnberg et al., 2003; Ohtake et al., 2003). Also, many PCB congeners and metabolites bind to ER and display “estrogenic” or “antiestrogenic” activity in bioassays (Connor et al., 1997; Ramamoorthy et al., 1997; Andersson et al., 1999; Routledge et al., 2000; Silva et al., 2002).

To assess possible adaptations in reproductive processes controlled by ER- and/or AhR-mediated mechanisms in wild NBH killifish, we sought to clone and characterize killifish cytochrome P450 aromatase (P450arom) isoforms, for use as markers of estrogen and dioxin effects. Endogenous estrogen biosynthesis, and thus, the availability of ligand for ER binding and transactivation, is controlled by the P450arom enzyme complex, a product of the *cyp19* gene. Although *cyp19* is found as a single copy in the haploid human genome (Simpson et al., 1994), studies in this laboratory have determined that fish have multiple structurally and functionally distinct P450arom isoforms, which derive from separate gene loci (*cyp19A* and *cyp19B*); are differentially expressed in brain (P450aromB \gg P450aromA) and ovary (P450aromA \gg P450aromB); and have a different constitutive expression (B \gg A) and developmental program (Gelinas et al., 1998; Tchoudakova and Callard, 1998; Kishida and Callard, 2001). Whereas P450aromB (but not -A) is upregulated by hormonal estrogen (estradiol) and xenoestrogens (diethylstilbestrol, bisphenol-A), P450aromA (but not -B) is downregulated by dioxin (Kishida et al., 2001; Novillo & Callard, unpublished data). In vivo effects are consistent with the presence of ER binding motifs (estrogen responsive elements, ERE) in the *cyp19B* promoter and two potential dioxin response elements (AhR/Arnt

binding motifs) in the *cyp19A* promoter, implying that each of these genes is a primary target of a subset of EDCs. Thus, without a priori knowledge of bioavailability, accumulation, metabolism or receptor binding characteristics, P450aromB and -A mRNAs are convenient markers of EDC effect, and provide an entry point for uncovering EDC-mediated perturbations of reproduction and development.

2. Materials and methods

2.1. Oligonucleotides

Oligonucleotides (Invitrogen Corp., Carlsbad, CA) used as PCR primers or hybridization probes are listed in Table 1. Oligonucleotides a1-9 and b1-8 were primers used in our PCR cloning strategy for P450aromA and -B cDNAs, respectively. Oligonucleotides a10-11 and b9-10 were used for semiquantitative reverse transcription (RT)-PCR; a12 and b11 as hybridization probes; and a15-16 and b14-15 for real-time quantitative (Q)PCR. Primers a1-3, a5, b1-3, and b5 were degenerate or non-degenerate primers based on conserved residues in previously reported teleostean brain and ovarian P450arom cDNAs. All other oligonucleotides were gene-specific, based on sequences of our newly isolated killifish P450arom cDNAs. Aromatase-specific real-time PCR primers were designed using PrimerExpress 2.0 (Applied Biosystems, Foster City, CA). All other aromatase primers were designed using the Primer3 program (Rozen and Skaletsky, 2000). RT-PCR and QPCR primers were validated using authentic aromatase cDNAs and tissue samples. Oligonucleotides n1-2 and n4 for semiquantitative and real-time PCR amplification of killifish actin were previously reported (Meyer et al., 2002; Garcia-Reyero et al., 2004). The actin probe n3 used for PCR and hybridization was based on GenBank sequence #AF397164. Vitellogenin primers (v1-2) used for real-time PCR have been described (Garcia-Reyero et al., 2004). Killifish *cyp11A1* primers (c1-2) for RT-PCR were first reported by Powell et al. (2000).

2.2. Collection sites

NBH, our polluted site, is on Buzzard's Bay approximately 55 miles south of Boston. From the 1940s

until 1978 it housed two capacitor manufacturing facilities that discharged PCBs and heavy metals both directly into the harbor and through the city's sewer system (Weaver, 1983). An EPA investigation of the harbor in 1976 found 18,000 acres of PCB contamination with concentrations in sediment in some areas exceeding 100,000 ppm (Weaver, 1983), in addition to elevated levels of cadmium, copper, chromium, and lead (Black et al., 1998b). In 1982, NBH was designated a Superfund site. The NBH killifish near our collection site has been found to have total PCB concentrations of 33,977 ng/g liver dry weight (Black et al., 1998b). Scorton Creek (SC), our reference site, is located on Cape Cod Bay in Sandwich, MA, approximately 30 miles northeast of NBH and in the same climatic zone. This collection site has previously been used as a reference location for killifish research (Bello et al., 2001).

2.3. Fish and tissue sampling

Adult male and female killifish approximately 5.3–9.4 cm in length were collected monthly May–October from SC using minnow traps. On the same day, fish of the same size range were obtained from the NBH Superfund site in collaboration with the USEPA National Health and Environmental Effects Laboratory, Atlantic Ecology Division, Narragansett RI. Within 12 h of capture, fish were anesthetized with 0.06% ethyl 3-aminobenzoate methanesulfonate salt (MS-222). After collection of blood from the caudal vein into heparinized capillary tubes, the fish were killed by decapitation. Total weight, standard length, liver and gonadal weights were determined and used to compute gonadosomatic index (GSI), hepatosomatic index (HSI), and condition index (CI; $CI = (\text{weight} \times 100) / \text{Length}^3$). For RNA analysis, brain, eye, gill, gonad, spleen, liver, heart, gut, muscle and abdominal fat (if present) were immediately frozen on dry ice and stored at -70°C no longer than 6 weeks. Additional samples of gonad and liver were fixed in 4% paraformaldehyde for paraffin embedding and standard hematoxylin–eosin staining. Opercula were collected to determine age.

2.4. RNA extraction

RNA was extracted from frozen tissues using Trizol (Sigma Aldrich St. Louis, MO), treated with DNaseI

(Roche Diagnostics Indianapolis, IN), size-separated on 1% agarose gels to assess quality, and yield determined spectrophotometrically. Where specified, poly A+ RNA was obtained from 100 µg of total RNA using a MicroPoly(A)Pure™ Kit (Ambion Inc. Austin TX).

2.5. PCR cloning strategy

Brain and ovarian RNA of SC fish were used for initial cloning of P450aromB and -A cDNAs, respectively. A stepwise PCR cloning strategy was employed essentially as described for goldfish (Tchoudakova and

Table 1
Oligonucleotide primers and hybridization probes

| Gene target | Oligo | Sequence | Nucleotide position | Reference |
|--------------|-------|-----------------------------|---------------------|-----------------------------|
| P450aromA | a1 | GTGCTGGAGATGGTGATCGC | 1004–1024 | |
| | a2 | TTGGTYTGTGGGAGGCAGTC | 1501–1521 | |
| | a3 | KCTGMTKCWGAAGATYCAHAARTA | 747–771 | |
| | a4 | A4 CCAGCTGAGCTTGAAGTAGATGT | 806–829 | |
| | a5 | CCAGCAACTAYTAYAAARCAART | 341–365 | |
| | a6 | TGCACCGGACAGAGTTCTCCACAA | 1307–1332 | |
| | a7 | TCCTCGCCGTTACTTCCAGCCATTC | 1371–1396 | |
| | a8 | TTTCCACAGCGCCACGTTATTGTTGA | 524–549 | |
| | a9 | TGAGGCCCTGCTGAGTATGAGCGTCT | 400–426 | |
| | a10 | CGACGAATTTAGTCTGGAGAACTT | 1335–1359 | |
| | a11 | TAAAGTCATGGTGCATTAAAGGTG | 1619–1643 | |
| | a12 | GTTACTTCCAGCCATTCGGCTCAGG | 1377–1402 | |
| | a13 | AATTTTACACTCTTCGGGTTTGAG | 3–28 | |
| | a14 | AAACCAACAAAGGGAAGAAATAAAC | 1837–1861 | |
| | a15 | TGGAGAGGAGACGCTCATACTCA | 391–414 | |
| | a16 | GGGAGGTGTAGTTTCCATTCTTCA | 441–465 | |
| P450aromB | b1 | CAGTGTGTGCTRGAGATGGTGWTC | 1019–1043 | |
| | b2 | CACCATRGCDATGTGCTTSCC | 1442–1463 | |
| | b3 | GACBCCTCWMCAYGTRGATG | 682–702 | |
| | b4 | TTCAAAGTAGTTGTGGATTTTC | 772–794 | |
| | b5 | GGAACAGCCTGTAAGTACTACAACAA | 338–364 | |
| | b6 | CCAGTCGCTTCTTCCAGCCCTTGG | 1396–1321 | |
| | b7 | TCTTGAAGAAGATATCAG | 819–837 | |
| | b8 | CTCGAGGCCTCTTTTGCTGCCAAATC | 469–495 | |
| | b9 | AACAGAAGTGAGAACTGGACGAC | 926–950 | |
| | b10 | TTCTTGATCTTCGTACCTTCGAC | 1274–1297 | |
| | b11 | GTTCTGGACCAAACAGCTGTGGAGA | 1141–1166 | |
| | b12 | GACAAACAGCAGCTACCAGAGAT | 120–143 | |
| | b13 | ATCAAGATGGCCTTCATCATCAC | 1458–1471 | |
| | b14 | ACGAGCACAGTCTGAGCATGAG | 1576–1598 | |
| | b15 | CTCAGATCCTCGTCATCGTTCA | 1638–1660 | |
| Actin | n1 | GGCCAACAGGGAGAAGATGACCCAGAT | NA | Meyer et al. (2002) |
| | n2 | GGATTCCGCAGGACTCCATTCCGA | NA | Meyer et al. (2002) |
| | n3 | CCGGTATCGTGATGGACTCT | NA | Garcia-Reyero et al. (2004) |
| | n4 | GCGTAAATACTCTGTCTGGATCGGAGG | NA | |
| | n5 | GTTTGAAGCATTTGCGGTGGACG | NA | |
| Vitellogenin | v1 | GAGGATCTGTGCTGATGCAGTTGTG | 3875–3900 | Garcia-Reyero et al. (2004) |
| | v2 | GGGTAGAAGGCAGTCTTTCCAGG | 4022–4046 | Garcia-Reyero et al. (2004) |
| P450 1A1 | c1 | ACCTGCCTTTCACAATCCCACTGCTC | 1294–1322 | Powell et al. (2000) |
| | c2 | TCGTTTCGTGCGATAACCTCACCGATG | 1526–1553 | Powell et al. (2000) |

Except where referenced, oligonucleotide sequences were designed in this laboratory. Position of targeted sequences is based on our newly isolated P450aromA and -B cDNAs (GenBank #AY428665 and #AY428666, respectively), or on previously reported GenBank entries (vitellogenin U07055: *cyp1A1* AF026800; actin AF435092); NA, not available.

Callard, 1998) and zebrafish (Kishida and Callard, 2001). Synthesized cDNA was amplified using 2 μ M of each primer (degenerate primer sets a1/a2 and b1/b2 for P450aromA and -B, respectively) with the following conditions: 94 °C/5 min; 35 cycles of 94 °C/30 s, 57 °C/30 s, 72 °C/1 min; and 72 °C/10 min. Amplified products were isolated from the gel using a Montage gel extraction kit (Millipore Corp, Billerica, MA) and cloned as described previously. Additional sequence was obtained using primer pairs a2/a3 and a4/a5 (P450aromA), and b2/b3 and b4/b5 (P450aromB). The amplification parameters were the same as used for the initial PCR except the annealing temperature was 56 °C. Five clones of each PCR product were sequenced in both directions. This and all subsequent sequence analyses were performed on an ABI PRISM® 3730 DNA Analyzer by Macrogen Inc. (Seoul, Korea).

The 3' end of each cDNA isoform was obtained by rapid amplification of cDNA ends (RACE) using a Generacer kit with Platinum *Taq* (Invitrogen), and primers a6 (P450aromA) and b6 (P450aromB). The ovarian product was reamplified using the nested primer approach and primer a7. Products were size-separated on a 1% agarose gel and purified using a SNAP column (Invitrogen). The purified products were TA cloned into pCR®4-TOPO® cloning vector (Invitrogen), and six clones of each were sequenced in each direction.

The 5' end of the P450aromA cDNA was obtained using the Generacer kit and 200 ng of polyA+ ovarian RNA. PCR amplification was carried out as described above using primer a8, and the SNAP-purified product was reamplified using the nested primer a9. The product was TA cloned into the TOPO TA vector and five clones were sequenced in each direction. The 5' end of the P450aromB cDNA was obtained using the 5'-RACE System, version 2.0 (Invitrogen) starting with 5 μ g brain RNA and primer b7 for reverse transcription. The resultant cDNA was amplified using the primer provided and gene-specific primer b8. The amplified product was separated on an agarose gel, purified using a SNAP column, and TA cloned into the pGEM T-Easy vector. Six clones were sequenced.

cDNA sequences were assembled using the contig program in Vector NTI (Invitrogen). All segments overlapped to varying degrees beyond the sequences targeted by the primers. Sequence was confirmed by direct sequencing of end-to-end PCR products

that were obtained using gene-specific primers pairs a13/a14 and b12/b13 which targeted sequences in the 5'- and 3'-UTRs. Five end-to-end PCR reactions were sequenced for P450aromA and five for P450aromB. Further, the five PCR reactions of each isoform were pooled separately, TA cloned into the pGEM-T-Easy vector, and four clones of each were sequenced. To assess possible differences in the coding regions of P450aromA and -B of SC and NBH fish, and to validate our choice of primers for RT-PCR and QPCR analysis of tissues from both populations, ovarian and brain RNAs from six NBH fish were reverse transcribed and amplified with primer sets a13/14 and b12/13; products were directly sequenced. To differentiate PCR error from polymorphisms, multiple independent PCR reactions were performed on cDNAs in which a possible polymorphism was identified.

2.6. Sequence comparisons and phylogenetic analysis

Deduced amino acids of newly isolated killifish P450aromA and -B (respectively, GenBank # AY428665 and # AY428666), and previously reported vertebrate aromatases (for GenBank accession numbers, see legend to Fig. 1), were aligned using ClustalX 1.81 (Chenna et al., 2003) and a Gonnet scoring matrix. Neighbor joining and parsimony trees were constructed in Phylip 3.6 (Felsenstein, 1993) using all characters and default parameters. Heuristic methods including 1000 bootstrap replicates in branch-swapping and tree-bisection reconnection in PAUP (Phylogenetic Analysis Using Parsimony, v4.02b) (Swofford, 2003) were used to obtain bootstrapping values (confidence figures at branch points) and to confirm topology. A consensus tree was produced using Phylip 3.6 (Felsenstein, 1993).

2.7. Semiquantitative RT-PCR and Southern transfer analysis

cDNA was synthesized from 3 μ g of RNA as described above, and separate aliquots (5%) were amplified using gene-specific primer sets b9/10 (P450aromB), a10/11 (P450aromA), n1/2 (actin), and c1/2 (*cyp1A1*). The reaction was heated to 94 °C for 10 min and subsequently amplified for 25 (P450aromB), 28 (P450aromA), 24 (*cyp1A1*) or 24

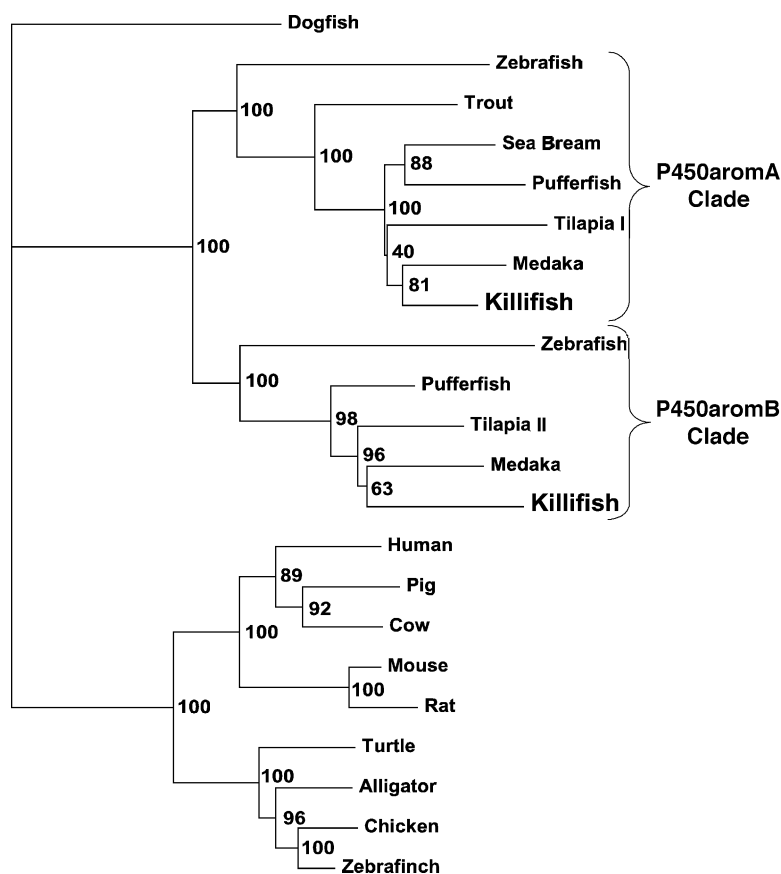


Fig. 1. Phylogenetic tree of P450arom proteins. Deduced amino acid sequences of killifish P450aromA (GenBank #AY428665) and P450aromB (GenBank #AY428666) were aligned with aromatas of representative vertebrates, and analyzed to produce a consensus tree with dogfish shark as the designated outgroup. Additional GenBank accession numbers used were: AAG35637 (dogfish shark); AAK00642 (zebrafish P450aromB); AAK00643 (zebrafish P450aromA); BAA11657 (medaka P450aromA); AAP83449 (medaka P450aromB); 228574 (trout P450aromA); AAO62626 (tilapia II, P450aromB); AAO62625 (tilapia I, P450aromA); AAL27699 (sea bream, P450aromA); BAA90529 (frog); AAG09376 (turtle); AAK31803 (alligator); AAA48738 (chicken); AAB32404 (zebra finch); BAA00551 (mouse); AAA41044 (rat); AAA62244 (cow); AAB51387 (pig Type3-placental); and AAA52132 (human). The deduced protein sequences for pufferfish P450aromB (FRUP00000132385) and P450aromA (FRUP00000165318) were obtained from the *Fugu* genome v3.0 database (Aparicio et al., 2002)(<http://genome.jgi-psf.org/fugu6/fugu6.home.html>) and extended using the three frame translation feature where possible.

(actin) cycles at 94 °C for 30 s (denaturation), 58 °C for 30 s (annealing), and 72 °C for 1 min. The last extension was 10 min. Amplified products were separated on 1.5% agarose gels, transferred to BrightStar[®]-Plus nylon membrane (Ambion) by upward capillary transfer, and crosslinked with a FB-UVXL-1000 UV crosslinker (Fisher Scientific, Hampton, NH). To increase sensitivity and specificity of product detection, hybridization probes were constructed by end-labeling oligonucleotides b11 (P450aromB), a12 (P450aromA) and n3 (actin) with [γ^{32} P] ATP and T₄ polynucleotide

kinase (Promega Corp.). Hybridization was carried out overnight at 60 °C in hybridization solution (5× SSC, 0.1% SDS, and 100 µg/ml sheared salmon sperm). The membranes were washed twice for five min at room temperature in 6× SSC and then in 6× SSC, 0.1% SDS. Autoradiograms were exposed for 15 min–2 h.

2.8. Real-time QPCR analysis

QPCR was performed on an ABI PRISM 7900 (Applied Biosystems) instrument. Reverse transcribed

RNA, prepared as described above, was diluted five-fold. Reactions were set up such that all samples for analyses of target and control mRNAs were on the same plate. Primers were a15/16 and b14/15 (P450aromA and -B); n3/4 (actin); and v1/2 (vitellogenin). The final reaction contained 50% Sybr Green Master Mix (Applied Biosystems), 1 μ M of each primer (0.5 μ M for actin, 0.25 nM for vitellogenin), and separate aliquots (3%) of cDNA. Four independent biological samples, each in triplicate, were analyzed for each tissue-type, gene, and treatment group. Amplification was performed under default conditions with the addition of a denaturing step to confirm the presence of a single amplicon. Data were analyzed using the Q-gene package and normalized to actin after correcting for differences in amplification efficiency as recommended in the Q-gene package using a dilution series for the amplification curve (Simon, 2003).

2.9. Vitellogenin and steroid immunoassay

Blood was centrifuged at $8000 \times g$ for 15 min at 4 °C and plasma transferred to Eppendorf tubes for storage at –70 °C until analysis. Vitellogenin protein was quantified by ELISA in plasma from individual male and female NBH and SC killifish using a killifish-specific antibody, (a service provided by the Molecular Biomarkers Core Facility, Biotechnology Program, University of Florida (Bowman et al., 2000)). Total androgen and estrogen were assayed in duplicate in plasma pools (60–180 μ l; 8–10 fish per pool; 3 pools for each sex and collection site during the breeding season). Radioimmunoassay procedures were as described previously using antibodies provided by Dr. G. Niswender (Colorado State University, Fort Collins, CO) (Rosenblum et al., 1985; Pasmanik and Callard, 1988). Cross-reactivity of each of the antibodies was as follows: androgen antibody (T > DHT) (Korenman et al., 1974); estrogen antibody (estradiol > estriol > estrone) (Gay and Kerlan, 1978). Values were expressed as pg steroid per ml plasma.

2.10. Age determination

Age was determined by counting opercular rings (Six and Horton, 1977). In brief, opercula were boiled in water for ~15 min, excess tissue was removed with forceps, and samples were stored in 70% ethanol no

longer than one month. Annual rings were counted in the preopercle component under a dissecting microscope.

2.11. Statistics

Statistical analysis was performed using the Sigma-Stat 2.0 package (Jandell Scientific). Normality and equal variance of the data set were verified. Data sets which passed both tests (HSI, P450aromB monthly expression, female vitellogenin mRNA, androgen RIA) were analyzed by ANOVA with all pairwise comparisons performed by the Tukey Test. Data which failed either the normality or equal variance tests were transformed using the arcsinh (male vitellogenin ELISA), log (female vitellogenin ELISA, male vitellogenin mRNA, estrogen RIA) or arcsine (GSI, CI, P450aromA monthly) methods and equal variance and normality were checked. None of the transformations produced data that passed normality and equal variance tests. The original data sets were then analyzed by the Kruskal–Wallis ANOVA on ranks method using Dunn's method for all pairwise comparisons. Significance was set at $P \leq 0.05$.

3. Results

3.1. Isolation of killifish P450aromA cDNA

P450aromA cDNA was cloned from ovaries of SC killifish in five overlapping segments. An initial fragment of approximately 500 bp was amplified using RT-PCR with primers a1/a2. This fragment was extended through separate RT-PCR reactions using degenerate primers (a3 and a5) together with gene-specific primer a4 to yield a 1179 bp sequence. The 5' and 3' ends were obtained by RACE with gene-specific primers (a6–9), yielding products of 522 and 723 bp, respectively. Database searches showed each segment to have a high level of identity to previously isolated P450aromA cDNAs. The assembled fragments resulted in a 1998 bp cDNA with a 1551 bp open reading frame (ORF) that encoded a full-length aromatase protein of 517 aa. The consensus nucleotide and deduced protein sequences can be obtained from GenBank (#AY428665). Alignments of the sequence data showed 4 bp substitutions in the coding region and one additional in the 3'-

untranslated region (UTR). The first three changes, when present, were always found together in the same cDNA fragment, but none of the substitutions in the coding region altered protein sequence. The 5'-UTR was 71 bp, but there was a second possible initiation site 30 bp downstream of the first.

3.2. Isolation of killifish *P450aromB* cDNA

A cDNA encoding a full-length *P450aromB* polypeptide was obtained by RT-PCR from the brain of SC killifish. An initial ~450 bp fragment was cloned using degenerate primer set b1/b2, and extended in two further PCR reactions using degenerate primers b3 and b5 together with gene-specific primer b4. The 1166 bp cDNA assembly obtained from the degenerate PCR reactions was extended by RACE with gene-specific oligonucleotides (b6, b4 and b7), which resulted in 5'- and 3'-RACE products of 760 and 752 bp, respectively. The final *P450aromB* cDNA sequence was 2279 bp, with an ORF of 1497 bp that encoded a 499 aa polypeptide. The consensus nucleotide and deduced protein sequences of the assembled fragments have been entered in GenBank (#AY428666). Both the 5'-UTR (141 bp) and the 3'-UTR (641 bp) of *P450aromB* were longer than the killifish *P450aromA* cDNA (respectively, 71 and 376 bp). Among the clones sequenced, 15 nt substitutions were identified in the coding region, eight of which had no effect on protein sequence. Four of the substitutions that affected protein sequence (nt 436, 748, 895–896, and 923) were conservative, resulting in the following amino acid changes: Y99H, L203P, N252S and L261I. Two additional non-conservative changes at bp 481 and 1583 resulted in the following substitutions: E114K and H481P, respectively. None of these substitutions occurred in putative functional domains.

3.3. *P450arom* cDNAs in NBH killifish

To assess possible site-specific polymorphisms in the coding sequence of killifish aromatases, RNA extracts were prepared from the brain and ovaries of six females from the NBH population, and used to obtain cDNAs by end-to-end PCR. Direct sequencing of all PCR reactions gave a consensus sequence for each of the two isoforms that was essentially the same as *P450aromB* and -A cDNAs of SC fish. More-

over, of the nucleotide substitutions found in NBH fish, all were found in the SC population (see above), but none occurred in regions targeted by RT-PCR or QPCR primers.

3.4. Sequence comparisons and phylogenetics

Alignment and analysis of the killifish *P450arom* sequences with those of other vertebrates produced a phylogenetic tree that was consistent with two distinct aromatase clades (A and B) in the teleostean lineage (Fig. 1), as initially reported by Tchoudakova and Callard (1998). All other vertebrates clustered in a third *P450arom* clade. It is noteworthy here that the dogfish aromatase, when not designated as the outgroup taxon, segregated with the non-teleostean clade (Fig. 2).

Both phylogenetic analysis and direct sequence comparisons show that killifish *P450aromA* and -B proteins are more closely related to medaka A- and B-aromatases (84.1 and 78.3% overall identity, respectively) than to each other (59.5%). Even within conserved functional domains, the two killifish isoforms have a lower sequence identity than the percentage identities obtained by comparison to their medaka counterparts (Table 2). Moreover, within the aromatase-specific region (presumed androgen binding domain), each of the killifish *P450aromA* and -B sequences had a higher degree of relatedness to the single human aromatase (respectively, 87 and 70%) than to each other (61%). The cloned killifish cDNAs confirmed several *P450aromA* versus *P450aromB*-specific features previously identified by comparison of fish aromatases (Kishida and Callard, 2001): i.e., an elongated membrane spanning domain in the teleostean *P450aromA* when compared to *P450aromB* or to non-teleostean aromatases; a serine residue at aa 91 in *P450aromA* instead of the conserved cysteine residue in *P450aromB* and in mammalian forms; a deletion of 4 aa at residue 196; and an elongated N-terminus and shortened carboxy end when *P450aromA* was compared to *P450aromB*.

Like all vertebrate cytochromes *P450*, killifish aromatases contain an absolutely conserved cysteine in the heme-binding domain (HBD), which is known to be essential for catalytic function (Graham-Lorence et al., 1995). When the core HBDs of selected vertebrate species were aligned, 7 of 13 positions were identical from fish to mammals, but a clear

| | |
|--------------|---|
| Stingray | FGPRSCVGVK [*] YVAM |
| Dogfish | CGPRSCVGVK [*] YIAM |
| Medaka B | CGPRSCVGVK [*] H [*] TIAM |
| Killifish B | CGPRSCVGVK [*] H [*] TIAM |
| Pufferfish B | CGPRSCVGVK [*] H [*] TIAM |
| Tilapia B | CGPRSCVGVK [*] H [*] TIAM |
| Sea Bass B | CGPRSCVGVK [*] Q [*] TIAM |
| Wrasse B | CGPRSCVGVK [*] H [*] TIAM |
| Trout B | CGPRSCVGVK [*] H [*] TIAM |
| Catfish B | CGPRACVGVK [*] H [*] TIAM |
| Grouper B | CGPRSCVGVK [*] H [*] TIAM |
| Zebrafish B | CGPRACVGVK [*] H [*] TIAM |
| Goldfish B | CGPRACVGVK [*] H [*] TIAM |
| Medaka A | SGPRACVGRH [*] TIAM |
| Killifish A | SGPRACVGVK [*] H [*] TIAM |
| Pufferfish A | SGPRACVGVK [*] H [*] TIAM |
| Sea Bream A | SGPRACVGVK [*] H [*] TIAM |
| Tilapia A | SGPRACIGK [*] H [*] MIAM |
| Sea Bass A | SGPRACVGVK [*] H [*] TIAM |
| Wrasse A | SGPRSCVGVK [*] H [*] TIAM |
| Trout A | SGPRSCVGVK [*] H [*] TIAM |
| Catfish A | SGPRSCVGVK [*] H [*] VIAM |
| Grouper A | SGPRSCVGVK [*] H [*] TIAM |
| Zebrafish A | SGPRSCVGVK [*] H [*] TIAM |
| Goldfish A | SGPRSCVGVK [*] H [*] TIAM |
| Turtle | FGPRGCVGVK [*] E [*] TIAM |
| Alligator | FGPRACVGVK [*] E [*] TIAM |
| Chicken | FGPRGCVGVK [*] E [*] TIAM |
| Zebra finch | FGPRSCVGVK [*] E [*] TIAM |
| Mouse | FGPRGCAGK [*] YTIAM |
| Rat | FGPRSCAGK [*] YTIAM |
| Pig | FGPRACAGK [*] YTIAM |
| Cow | FGPRGCAGK [*] YTIAM |
| Human | FGPRGCAGK [*] YTIAM |
| | ***. * * : . : ** |

Fig. 2. Sequence alignment of heme-binding domains (HBD) of vertebrate P450arom proteins. All teleostean species with reported P450aromA and -B sequences were included together with representatives of other vertebrate classes (see legend to Fig. 1 including stingray (AF097513). Identical and similar amino acid residues are marked by asterisks and dots, respectively. Boxed residues highlight phylogenetic patterns discussed in Section 3.

phylogenetic pattern emerged by inspection of three of the non-conserved positions. First, five residues toward the amino terminus from the conserved cysteine, all teleost fish B-forms had a second cysteine residue, whereas all A-forms had a serine in this position, and amphibian, reptilian, avian and mammalian aromatases had a phenylalanine. Second, all but one (sea bass B) of the A- and B-aromatases of teleost fish had a his-

tidine four residues downstream of the conserved cysteine, while birds and reptiles had a phenylalanine and mammals had a tyrosine in this position. Interestingly, dogfish also had a tyrosine, but stingray had a phenylalanine. Finally, with one exception (tilapia A), all non-mammalian aromatases had a valine after the conserved cysteine, while mammals had an alanine in this position.

3.5. Tissue-specific P450arom expression

Semiquantitative RT-PCR analysis, followed by hybridization of PCR products with internal gene-specific probes, was used to determine the tissue distribution of P450aromA and -B mRNAs in killifish populations. A tissue series from SC fish collected in May is shown in Fig. 3 and represents results obtained from four independent tissue series for each collection site and sex. P450aromB was expressed predominantly in the brain, with lower amounts in the eye and ovary. P450aromA was expressed predominantly in the ovary with much lower amounts in brain and eye (retina). Expression patterns in non-gonadal tissue were essentially the same in SC and NBH fish, with one exception. NBH fish sometimes had abdominal fat that was never found in SC fish. When measured in fat, authentic P450aromA was detected at low levels (data not shown). Using this highly sensitive and specific method of analysis, we were unable to detect aromatase expression in killifish gill, testis, spleen, liver, heart, gut, or muscle. Analysis by QPCR allowed direct comparison of P450aromA and -B normalized to actin in the brain, eye, and ovary, and additionally revealed a low level of P450aromA mRNA in abdominal fat (Table 3).

3.6. Site, sex and seasonal differences in P450aromB and -A expression

As shown in Fig. 4, when expressed levels of P450aromB (an established marker of neuroactive estrogen) were analyzed in reproductively active fish by three-way ANOVA, significant sex ($P \leq 0.001$; $F = 20.882$), site ($P \leq 0.001$; $F = 59.146$) and seasonal ($P \leq 0.001$; $F = 71.751$) differences were obtained. Regardless of reproductive status or sex, brain levels of P450aromB were elevated approximately two-fold in NBH as compared to SC fish. At a given site, there

Table 2

Comparison of conserved functional domains of P450aromA and -B isoforms within and across species

| % Identity | Killifish A | | | | Killifish B | | | |
|--------------------|-------------|----------|----------|-------|-------------|----------|----------|-------|
| | Killifish B | Medaka A | Medaka B | Human | Killifish A | Medaka A | Medaka B | Human |
| Overall | 60 | 84 | 55 | 49 | 60 | 58 | 78 | 51 |
| I-helix | 88 | 97 | 91 | 70 | 88 | 91 | 97 | 64 |
| Aromatase-specific | 61 | 87 | 61 | 87 | 61 | 61 | 83 | 70 |
| Heme-binding | 86 | 93 | 86 | 71 | 86 | 79 | 100 | 71 |

To determine percentage identities, deduced amino acid sequences were aligned, and the boundaries of functional domains determined using the human P450arom protein as template: I-helix (residue 291–324); aromatase-specific (residue 379–402); heme-binding (residue 431–444) (Simpson et al., 1994).

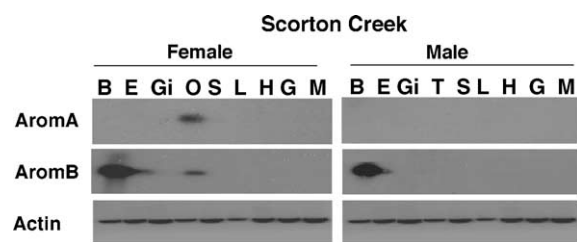


Fig. 3. Tissue-specific expression of P450aromA and -B in male and female killifish. Tissues were collected from reproductively active fish populations in SC and NBH (during May). For each RNA extract, like-tissues were pooled from two males and two females fish. Total RNA (3 µg) was analyzed by RT-PCR/Southern Transfer. Results are representative of a total of four independent tissue series for each sex and collection site. Brain (B), eye (E), gill (Gi), testis (T), ovary (O), spleen (S), liver (L), heart (H), gut (G), and muscle (M).

was a sex difference during the period of reproductive activity, with females having approximately two times higher brain P450aromB levels than males; however, sex differences were not evident in reproductively regressed fish, due mainly to a seasonal decline in aromatase expression in female brain with little or no change in males. Compared to the B-mRNA species in brain, A-mRNA levels were ~16 times lower when

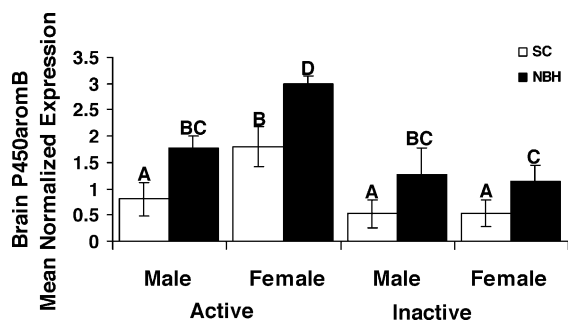


Fig. 4. Site-specific, sex and seasonal differences in brain P450aromB expression, as measured by real-time QPCR. Males and females were collected from SC and NBH in June and July (reproductive activity) and August and September (reproductive inactivity). Two RNA extracts, each comprising brains of two fish, was prepared from each sex, site, and time point and analyzed by QPCR. Data were analyzed by three-way ANOVA. When results were analyzed by month, no significant differences were obtained among reproductively active and inactive fish at a given site; therefore, monthly data were combined by site according to reproductive status. Each bar represents mean normalized P450aromB expression \pm standard deviation of four independent biological samples. Bars with different letters differ significantly ($P < 0.05$) as determined by the Tukey test.

Table 3

Values are, mean normalized expression ($\times 10^3$) as measured by QPCR of P450aromA and -B

| | P450aromA | | | | | P450aromB | | | |
|-------|-------------|---|---------------|---|----------------|-----------|----------------|-----|--|
| | SC | | NBH | | | SC | | NBH | |
| Brain | 1.8 ± 0.05 | A | 2.1 ± 0.06 | A | 1841.2 ± 45.34 | A | 2433.2 ± 15.37 | B | |
| Eye | 0.4 ± 0.12 | B | 0.4 ± 0.13 | B | 0.2 ± 0.02 | C | 0.3 ± 0.07 | C | |
| Ovary | 80.0 ± 1.55 | C | 123.2 ± 92.34 | C | 1.6 ± 0.66 | D | 1.7 ± 0.41 | D | |
| Fat | ND | | 4.5 ± 1.01 | D | ND | | ND | | |

Measured by QPCR of P450aromA and -B in brain, eye, ovary, and fat of reproductively active female killifish from SC and NBH \pm standard deviation ($n = 4$ per treatment tissue group; ND, not determined).

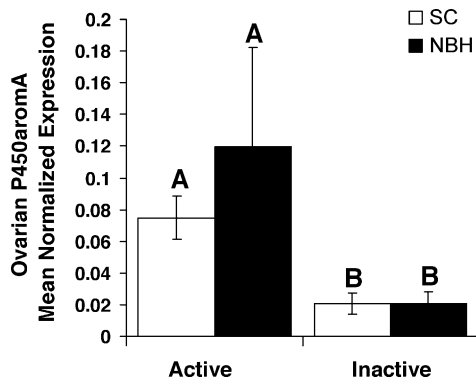


Fig. 5. Site-specific and seasonal differences in ovarian P450aromA expression, as measured by real-time QPCR. Fish were sampled as described in legend to Fig. 4. Each bar represents mean normalized P450aromA expression \pm standard deviation of four independent ovarian RNA pools, each from two animals. Bars with different letters differ significantly ($P < 0.05$) as determined by Kruskal–Wallis ANOVA on ranks with Dunn's method for pairwise comparisons.

both were expressed relative to actin in the same samples; however, because measurements were close to the limits of sensitivity of the QPCR assay, it was not possible to determine site, sex or seasonal variations in brain P450aromA with accuracy (data not shown).

One-way ANOVA on ranks showed that ovarian P450aromA expression was significantly affected by reproductive condition ($P \leq 0.001$) but did not differ in fish from the two collection sites (Fig. 5). Concomitant with reproductive regression, P450aromA expression in ovary declined to the same low levels at both sites. Compared to the A-isoform, ovarian P450aromB mRNA was ~ 50 – 100 times lower and highly variable; no significant effect of site or season was observed (data not shown).

3.7. Site, sex and seasonal differences in vitellogenin expression

Vitellogenin was measured as a second, independent marker of estrogen exposure and effect, and an indicator of reproductive condition. As shown in Fig. 6, results obtained by measuring vitellogenin as hepatic mRNA or as circulating protein essentially paralleled each other (compare panels A and B). When vitellogenin mRNA was analyzed in males by one-way ANOVA on ranks, significant site differences were found ($P \leq 0.001$), but no significant effects of repro-

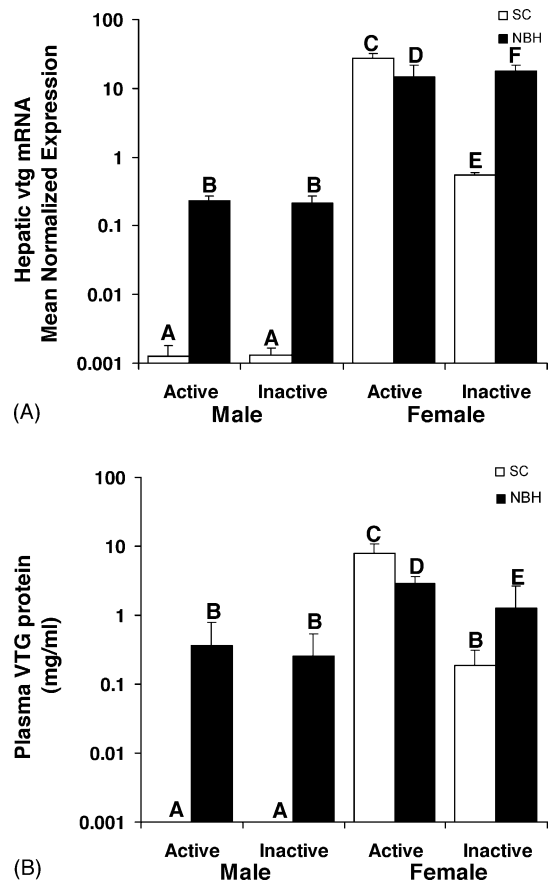


Fig. 6. Site, seasonal and sex differences in vitellogenin mRNA (A) and protein, (B) fish were collected as described in legend to Fig. 4. Vitellogenin mRNA was measured by real-time QPCR in liver of male and female killifish. Each bar represents mean normalized vitellogenin expression \pm standard deviation of four independent hepatic RNA pools (see legend, Fig. 4) plasma vitellogenin was measured by Elisa in individual male and female killifish. Each bar represents mean plasma vitellogenin (mg/ml) \pm standard deviation of four fish collected in June (reproductive activity) and August (reproductive inactivity). For statistical analysis see Section 3. Bars with different letters differ significantly ($P < 0.05$).

ductive status. When vitellogenin mRNA was analyzed in females by two-way ANOVA, significant effects of site ($P \leq 0.001$; $F = 43.680$) and reproductive condition ($P \leq 0.001$; $F = 26.804$) were observed. Similarly, results of plasma vitellogenin were significantly affected by site in males, as determined by analysis of variance on ranks ($P \leq 0.005$), but only females varied significantly by site ($P < 0.05$) and reproductive status ($P < 0.05$). In reference site (SC) males, vitellogenin

expression was low (mRNA) or undetectable (plasma protein), while in NBH fish vitellogenin expression was elevated >100-fold, and this was true whether or not the fish were reproductively active. As expected, reproductively active and inactive SC and NBH females all had much higher levels of vitellogenin mRNA and protein than males at the same site (all $P \leq 0.01$), when analyzed by paired Tukey test or Dunn's method. In marked contrast to males, vitellogenin mRNA and protein were depressed in NBH as compared to SC females during the breeding season but, following seasonal gonadal regression, mean vitellogenin levels declined to a lesser extent in NBH than in SC fish.

3.8. Tissue-specific *cyp1A1* expression

As an additional end point of pollutant exposure and effect, a DLC inducible marker (*cyp1A1*) was estimated by semiquantitative RT-PCR in reproductive and non-reproductive tissues of fish at both sites. As shown in Fig. 7, *cyp1A1* expression was similar in liver, brain, and gonads of reproductively active SC and NBH fish, but a more intense band was obtained in the eye (retina) of NBH males and females as compared to SC fish.

3.9. Physiological and reproductive status

To assess the general physiological condition and reproductive status of fish at the two collection sites,

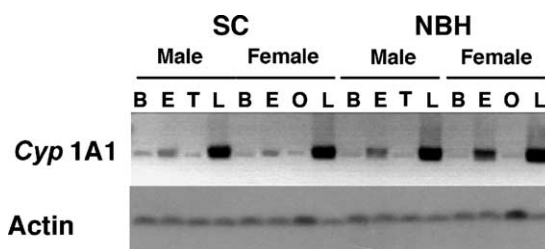


Fig. 7. Site and tissue-specific expression of *cyp1A1* as determined by semiquantitative RT-PCR analysis. Reproductively active fish were sampled as described in legend to Fig. 3. Total RNA (3 μ g) was analyzed by RT-PCR and amplified products visualized on ethidium bromide stained gels. Results are representative of two independent tissue series from each sex and collection site, brain (B), eye (E), testis (T), ovary (O), liver (L).

we determined GSI, HIS and CI. During the period of reproductive activity (May–July) and reproductive inactivity (August–October) parameters did not vary by month; therefore, monthly data were combined. Age (1.96 ± 0.27 year), length (7.13 ± 0.77 cm), and weight (7.53 ± 2.72 g) did not differ significantly by site, sex or reproductive condition, nor did the CI (1.9479 ± 0.28) differ by sex or site at any time of year. When GSI was analyzed by one-way ANOVA on ranks, there were significant effects of sex ($P < 0.05$) and reproductive status ($P \leq 0.001$) (Fig. 8A). These data confirm that fish collected between May and July were reproductively active while those from August–October was reproductively regressed. While no effects of site were

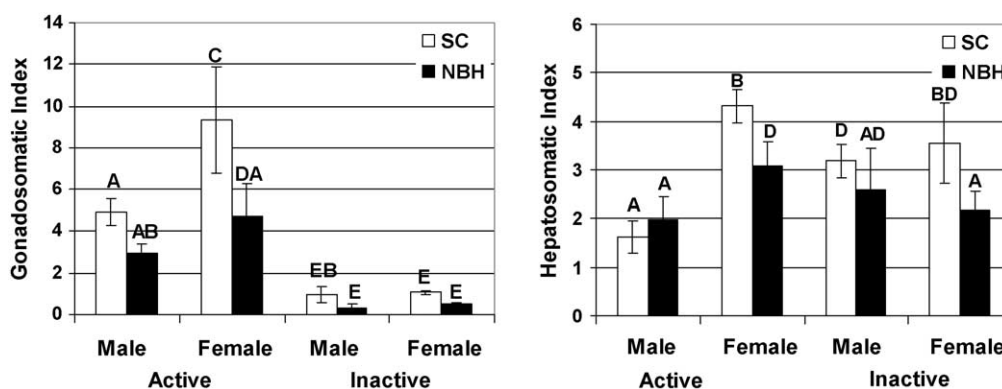


Fig. 8. Site, seasonal and sex differences in reproductive status measured as (A) gonadosomatic index (GSI) and (B) hepatosomatic index (HSI). Six fish of each sex were collected monthly between May and October from the populations at SC and NBH. When analyzed by month, no significant differences were obtained among reproductively active (May–July) and inactive (August–September) fish at a given site; therefore, monthly data were combined by site according to reproductive status. Each bar represents values (\pm standard deviation) from 18 (active) or 12 (inactive) fish. For statistical analysis see Section 3. Bars with different letters are significantly different ($P < 0.05$).

Table 4
Total androgen and estrogen in reproductively active (July) SC and NBH killifish

| | <i>n</i> ^a | Androgen | | | Estrogen | | |
|---------|-----------------------|----------|---------|--------------|----------|---------|--------------|
| | | pg/ml | ±S.E.M. | Significance | pg/ml | ±S.E.M. | Significance |
| Males | | | | | | | |
| SC | 3 | 2014.1 | 562.6 | A | 22.1 | 3.0 | A |
| NBH | 3 | 905.1 | 373.9 | B | 22.6 | 1.4 | AC |
| Females | | | | | | | |
| SC | 3 | 619.1 | 84.7 | C | 755.6 | 177.2 | B |
| NBH | 3 | 703.4 | 220.2 | BC | 318.0 | 144.2 | C |

Androgen levels varied significantly by site ($P \leq 0.05$) and sex ($P \leq 0.005$) as determined by two-way ANOVA. Estradiol showed significant variation by site and sex ($P \leq 0.025$) as determined by Kruskal–Wallis one-way ANOVA. Within each steroid category, values that differed significantly ($P \leq 0.05$) are indicated by different letters.

^a Values are the mean (±S.E.M.) of three plasma pools, each prepared from equivalent amounts of plasma from 8 to 10 fish.

found during reproductively senescent females or in males, NBH females had a significantly reduced GSI ($P < 0.05$) when compared to the reference site. By contrast, GSI of reproductively inactive fish was the same at both sites. HSI when compared by a one-way ANOVA with eight factors showed significant differences among the treatments ($P \leq 0.001$; $F = 23.676$) (Fig. 8B). Consistent with the male–female and seasonal differences in vitellogenin, pairwise comparisons using the Tukey test showed significant effects of site for all samples ($P < 0.05$), effects of sex only during the reproductive season ($P < 0.05$), and reproductive status only for females ($P < 0.05$). During the period of reproductive activity, females had higher HSI than males at both sites; however, the HSI of NBH females was low compared to SC fish. Seasonal reproductive regression was accompanied by an increase in HSI in males at both sites, but there was no change in SC females and a decrease in NBH females.

3.10. Plasma androgen and estrogen

As an additional index of reproductive condition, total plasma androgen and estrogen were measured during the breeding season in both populations (Table 4). A two-way ANOVA showed a significant effect of sex ($P < 0.005$; $F = 14.939$) and site ($P < 0.05$; $F = 6.152$) on plasma androgen. Males had higher androgen levels than females at both sites, but NBH males had <50% of levels in SC males. A one-way ANOVA on ranks showed that total circulating estrogen was significantly reduced in NBH females as compared to SC females ($P < 0.05$). Although mean plasma estrogen was lower

in males than in females at both sites, sex differences were significant only in the SC population ($P < 0.05$).

3.11. Histology

Results of gonadal histology confirmed the reproductive status and measured GSI of killifish collected at each site, and were essentially as described for NBH killifish by (Black et al., 1998b) (data not shown). While we observed a reduction of GSI in NBH females, which was not found by Black, we similarly observed no obvious differences in follicle size ranges (Black et al., 1998b). As an additional indicator of overall health and reproductive condition, H & E stained liver sections were examined. In the NBH population, both necrotic zones and cysts were identified, as previously described in killifish from Piles Creek, NJ (Schmalz et al., 2002). Neither cysts nor necrotic zones were found in any of the fish from SC. Overall, 88% of the NBH animals displayed hepatic abnormalities (data not shown).

4. Discussion

In this study, we successfully cloned and characterized P450aromA and -B cDNAs from killifish, and used sequence information to design primers and probes to quantify aromatase expression in brain and ovary as a measure of pollutant effect and reproductive status. While there are no apparent differences in coding sequences, or in overall tissue distribution patterns of aromatases from SC and NBH fish (P450aromB, brain \gg ovary; P450aromA, ovary \gg brain), QPCR

analysis reveals increased levels of P450aromB mRNA in the brain of the NBH, as compared to SC, killifish and a seasonal increase in brain levels in females, but not males, from each site. This finding, together with elevated levels of vitellogenin in male fish, suggests ongoing exposure and effects of estrogenic chemicals or metabolites in the NBH killifish. Further, despite the apparent reproductive and developmental success of NBH populations, after an estimated 15–20 generations in the highly polluted environment of a Superfund site, a number of molecular and physiological parameters are indicative of some degree of endocrine disruption of the HPG axis.

Although similar in size and organization to other teleostean aromatase cDNAs, the killifish ovarian form has a 5'-UTR that is 71 bp, which is longer than those of many fish P450aromA cDNAs: e.g., medaka, 39 bp (Watanabe et al., 1999). The 3'-UTR is 376 bp, longer than medaka (241 bp) and zebrafish (245 bp) but shorter than catfish (491 bp) P450aromA cDNAs (Trant, 1994; Watanabe et al., 1999; Kishida and Callard, 2001). Like other teleostean P450aromA sequences (Fukada et al., 1996; Kishida and Callard, 2001; Tchoudakova et al., 2001), there is a second ATG sequence 30 bp downstream of the first, but its use as an alternative initiation site in any species remains to be determined. The killifish brain form has a 5'-UTR (141 bp) that is intermediate in length when compared to catfish (193 bp) and medaka (106 bp) P450aromB cDNAs (Wang et al., 2003). The P450aromB 3'-UTR is 641 bp, which is longer than the killifish P450aromA cDNA but within the range of variation of other teleostean P450aromB 3'-UTRs (269–2200 bp) (Chang et al., 1997; Gelinas et al., 1998; Kazeto et al., 2001; Kishida and Callard, 2001; Choi et al., 2003; Wang et al., 2003; Blazquez and Piferrer, 2004; Li et al., 2004). Although trout has been reported to have a second, 5'-truncated P450aromB cDNA (Valle et al., 2002), no truncated 5'-RACE products were detected in our studies in killifish.

The deduced polypeptide sequences of the two killifish aromatases have a very high degree of sequence identity when compared to their counterparts in medaka (84 and 78% for P450aromA and -B, respectively), but only 60% identity when compared to each other, and the same intra- and cross-species relatedness is evident when sequences within conserved functional domains are compared. A plausible hypothesis is that selection pressures involved in optimizing tissue-specific func-

tions of duplicated *cyp19* genes became fixed in the teleostean lineage long before the divergence of present day fish species. Gaucher et al. (2004) have argued that a relatively recent duplication event in the peccary *cyp19* lineage could be linked to larger litter size and increased reproductive fitness of the lineage of contemporary pigs (Gaucher et al., 2004).

Additional insight into the evolution of vertebrate aromatase genes is revealed by analysis of the catalytically important heme-binding domains (HBD). HBD are highly conserved throughout the cytochrome P450 family (Graham-Lorence et al., 1995), and 7 of 13 residues are absolutely conserved among the vertebrate aromatases. Nonetheless, a clear phylogenetic and isoform-specific pattern emerges from inspection of three of the other six residues, suggesting that these substitutions were systematic, not random, over the course of evolution. Of note is a cysteine to serine change five residues upstream of the absolutely conserved cysteine when teleostean B- and A-forms are compared. First noted by (Kishida and Callard, 2001), the cloning of additional teleostean aromatases has reinforced the conclusion that this is an isoform-specific feature. Although the additional cysteine in teleostean P450aromB forms is also found in the single dogfish aromatase, the stingray aromatase has a phenylalanine in this position, characteristic of non-teleostean vertebrates eliminating a clear determination of the true ancestral form. When expressed in CHO cells, human and teleostean P450aromA and -B isozymes display different reaction kinetics and substrate and inhibitor characteristics which may be related to HBD substitutions (Zhao et al., 2001). Sequence differences among aromatases from different vertebrate species could be exploited as a useful alternative to site-directed mutagenesis for analysis of structure-function relations.

Consistent with previous reports in goldfish and zebrafish (Kishida and Callard, 2001; Tchoudakova et al., 2001), the two killifish aromatases are differentially expressed in brain (B > A) and ovary (A > B). Although P450aromB mRNA is reported to be present in peripheral tissues like the gill in trout (Valle et al., 2002), and P450aromA mRNA has been described in testis in sea bass (Dalla Valle et al., 2002), the highly sensitive and specific method of analysis used in our study (RT-PCR/Southern transfer) failed to detect aromatase mRNA in killifish gill, testis, spleen, liver, heart, gut, or muscle. Overall tissue expression patterns are the same

in SC and NBH fish, with one exception: low levels of P450aromA are detectable in abdominal fat, which was present in some but not all NBH fish. This finding is of interest because aromatase is expressed in fat in humans, and contributes significantly to the circulating estrogen pool in obese individuals (MacDonald et al., 1978). Nonetheless, fish collected from different sites within NBH vary in the occurrence and amount of abdominal fat (unpublished observation), suggesting that it is probably not related to pollution per se.

Real-time QPCR analysis of P450aromA and -B mRNAs, corrected for amplification efficiency of the two isoforms, enabled us to compare mRNA levels of each isoform when normalized to actin in different tissues, different fish populations, and males and females under different reproductive conditions. Tissue comparisons show that total aromatase mRNA (-B plus -A) in killifish brain is ~40-fold higher than in ovary, very similar to the ~50-fold difference observed between zebrafish brain and ovary (Sawyer et al., unpublished data) and in the same range as differences between catalytic activity between goldfish brain and ovary (10–100-fold) (Pasmanik and Callard, 1988). Whether the high levels of P450aromB mRNA in fish brain are a consequence of high rates of transcription, or are due to exceptional mRNA stability, remains to be tested. QPCR also shows a degree of overlapping expression of P450aromA and -B isoforms in killifish, as previously reported by semi-quantitative RT-PCR analysis in goldfish and zebrafish (Tchoudakova and Callard, 1998; Kishida and Callard, 2001). In zebrafish, the B-isoform (although low) is enriched relative to -A in unfertilized zebrafish eggs (1:10) when compared to the B:A ratio in ovaries (1:50) (Kishida and Callard, 2001; Sawyer and Callard, unpublished data). The implication is that P450aromB mRNA in fish ovaries is preferentially expressed in mature oocytes, which may be related to maternal transfer and an unidentified function in the zygote.

Comparison of killifish populations from polluted (NBH) and reference (SC) sites provides clear evidence of endocrine disruption, as measured by QPCR analysis of P450aromB and vitellogenin. Most notable are the relatively high levels of P450aromB mRNA in the brain of NBH male and female killifish, indicative of exposure to estrogenic pollutants or metabolites, most likely PCBs. This interpretation is supported by laboratory experiments showing that in vivo

treatment with estradiol or aromatizable androgen (but not 5 α -dihydrotestosterone, a non-aromatizable androgen) elevates brain aromatase activity and mRNA of gonadectomized or reproductively inactive goldfish (Pasmanik and Callard, 1988; Gelinas et al., 1998), and with experiments showing that estradiol, bisphenol-A and diethylstilbestrol induce a dose-related increase in P450aromB expression in zebrafish embryos (Kishida and Callard, 2001; Kishida et al., 2001). Additionally, in goldfish, seasonal cycles of brain aromatase activity and mRNA, with a peak at spawning, have been ascribed to an autoregulatory feedback loop driven by the product of aromatization (estrogen) (Pasmanik and Callard, 1988; Gelinas et al., 1998). It is significant here that P450aromB is elevated two- to three-fold in both SC and NBH females in seasonally active versus inactive fish despite site differences in actual values. These findings suggest that SC and NBH killifish respond similarly to seasonal increases and decreases in gonadal estrogen and/or androgen (precursor). Paradoxically, male killifish do not display seasonal changes in brain P450aromB mRNA, which may reflect differences in gonadal steroid production or indicate sex differences in neural responsiveness to endogenous estrogen.

Results described above are consistent with a report describing elevated brain aromatase activity in mosquitofish living downstream of a paper mill (Orlando et al., 2002), but in another study, brain aromatase activity was lower in trout exposed to an unknown endocrine disrupting chemical (Noaksson et al., 2003). Discrepant results in wild fish populations could be due to many different factors, including the nature, concentration and duration of exposure to a given pollutant, and/or to chemical effects on the functioning of the HPG axis rather than accumulated levels of aromatase mRNA or enzyme protein per se.

The idea that elevated levels of P450aromB in brain of NBH fish signify exposure to estrogenic EDC is further supported by elevated levels of vitellogenin in reproductively active and inactive males, and in reproductively inactive females from the same site. Vitellogenin is a female-specific yolk protein required for folliculogenesis and egg production, and is normally controlled by hormonal estrogen acting via hepatic ER. The presence of vitellogenin mRNA and protein in males of many different teleostean species is accepted as a reliable indicator of estrogenic contaminants in aquatic environments (reviewed in Sumpter

and Jobling, 1995; Matthiessen and Sumpter, 1998; Monteverdi and Di Giulio, 2000). In contrast to elevated vitellogenin expression in males and inactive females, however, vitellogenin expression is lower in reproductively active females from NBH when compared to SC females. These data together with a reduced GSI, lower levels of plasma estrogen, and a decreased HSI, in NBH females indicate impairment of the HPG–liver axis. Although NBH males have reduced levels of circulating testosterone, GSI is not significantly affected. It should be noted that disruption of the HPG axis can occur, not only through feedback effects of pollutants at the level of the hypothalamic–pituitary complex, but also by effects on gonadal steroidogenesis directly (DiBartolomeis et al., 1986; DiBartolomeis et al., 1987; Goldman and Yawetz, 1990; Moore et al., 1991; Goldman and Yawetz, 1992; Kovacevic et al., 1995).

As regards ovarian P450aromA in reproductively active killifish from the two sites, results are difficult to interpret. Experiments with zebrafish embryos show that P450aromA expression is unaffected by estrogen or xenoestrogens (Kishida and Callard, 2001; Kishida et al., 2001), but mRNA levels decrease in a dose-dependent manner after dioxin exposure (Novillo and Callard, unpublished data). Although we predicted low levels of ovarian P450aromA in NBH killifish, expression does not differ significantly in ovaries of fish from the two sites. One explanation is that resistance to AhR ligands in NBH populations attenuates effects of DLC on ovarian P450aromA expression but estrogenic pollutants in the NBH environment could affect the ovary indirectly or via the HPG axis and thereby confound results.

In contrast to molecular and physiological indices of estrogen-mediated endocrine disruption in NBH fish, the prototypical marker of AhR activation (hepatic P4501A1) shows decreased inducibility in NBH as compared to SC fish (Bello et al., 2001), a result interpreted as resistance to effects of AhR ligands (Powell et al., 2000; Bello et al., 2001; Nacci et al., 2002). Interestingly, in two independent experiments, P4501A1 is elevated in the eye of NBH as compared to SC fish, suggesting that sensitivity/responsiveness of AhR signaling pathways, and the mechanisms involved in acquired resistance, may be tissue-specific.

Finally, it is tempting to suggest that observed changes in mechanism-based molecular markers such

as P450aromB and -A are directly or indirectly related to reproductive deficits seen in this report, and the high female, embryo and larval mortality reported previously (Black et al., 1998b). However, the role of factors such as parasite burden (Schmalz et al., 2002) and a consequent reduction in hepatic function should not be underestimated, nor can we absolutely rule out site-specific differences such as temperature and nutrition (Gleason and Nacci, 2001).

5. Conclusion

Despite long term, multigenerational exposure to pollutants at the NBH Superfund site, and effects described in this and many related studies, it is remarkable that the NBH population continues to maintain seasonal spawning patterns and is capable of survival, reproduction and development. This assumes relatively normal gamete production, spawning behavior, and functioning of the HPG–liver axes and other systems. It also implies that gonadal sex determination, and growth and development of the reproductive system of embryos, larvae and juvenile fish, all operate within an acceptable range. Should we conclude, therefore, that EDC have effects on aromatase levels but no ultimate consequences for reproduction? Or, are fish in the present day NBH population the beneficiaries of selection pressures that attenuated or neutralized the adverse effects of estrogenic pollutants? If so, and by analogy to acquired resistance of killifish populations to AhR ligands, it is plausible that the NBH population has acquired a partial genetic or physiological resistance to estrogen signaling. Research addressing this hypothesis is in progress.

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